# STIC-ILL

**From:** Baskar, Padmavathi
**Sent:** Monday, June 23, 2003 12:33 PM
**To:** STIC-ILL
**Subject:** 09/300612, please consider this as rush


Bone "The Pathogenesis of Sepsis" Ann Int Med 115(6) 457-469 1991.
Guyton "Textbook of Medical Physiology" 8th Ed 269-271 1991.
Mousstach e et al "Mechanisms of Resistance of the Opossum to Some Snake Venoms" Toxicon 17(Suppl. 1) 130 1979.
Domont et al. "Natural Anti-Snake Venom Proteins" Toxicon 29(10) 1183-1194 1991.
Perales et al "Neutralization of the Oedematogenic activity of Bothrops Jararaca venom on the Mouse Paw by an antibothropic
Fraction Isolated from Opossum·serum" Agents Actions 37(3-4) 250-259 1992.
Tomihara et al. "Purification of Three Antihemorrhagic Factors From The Serum of A Mongoose" Toxicon 25(6) 685-689 1987.
Perates et al. "Anti Snake Venom Protein from Didelphidae" Abstract 10th World Congress. Toxicon 30(5-6) 543 1992.
Menchaca et al. "The Purification & Characterization of An Antihemerrhagic Factor in Opossum Serum" Toxicon 19(5) 623-632 1981.

Toxicon 14(4) 337-340, 1976

Tarng et al , Toxicon 24(6) 567-573, 1986.

Toxicon 34(11-12) 1313-6, 1996.

**Toxicon 36(10) 1451-9, 1998.**
**HIGH TECH SEPARATIONS NEWS 1996, V9,N4, SEP1996**

Toxicon 37(6) 949-954, 1999.

Toxicon 37(5) 703-728, 1999.

biochimica et biophysica acta 1995, 1245 (2) 232-8

Padma Baskar
Art Unit 1645
Patent Examiner/Biotechnology
CM-1, 8E-13
703-308-8886

1

# REVIEW ARTICLE

# NATURAL ANTI-SNAKE VENOM PROTEINS*

G. B. Domont,[1] J. Perales[2] and H. Moussatché[3]

[1]Departamento de Bioquimica, Instituto de Química, UFRJ, 21910, Rio de Janeiro, RJ, Brazil; [2]Escuela de Medicina, Universidad Centro Occidental, Barquisimeto, Venezuela; and [3]Departamento de Fisiologia e Farmacodinâmica, Instituto Oswaldo Cruz, RJ, Brazil

G. B. Domont, J. Perales and H. Moussatché. Natural anti-snake venom proteins. *Toxicon* 29, 1183–1194, 1991.—The resistance of several animals to snake venom has been reviewed. Some general concepts are introduced to allow the comparative evaluation of the resistance of different animals studied by different investigators. The purification and properties of several factors isolated from the serum of different animals by some researchers are described: *Trimeresurus flavoviridis* (Omori-Satoh *et al.*, 1972); *Vipera palaestinae* (Ovadia *et al.*, 1975, 1977); *Sigmodon hispidus* (Pichyangkul and Perez, 1981); *Didelphis virginiana* and *Didelphis marsupialis* (Menchaga and Perez, 1981; Moussatché *et al.*, 1979, 1980, 1981; Perales *et al.*, 1986, 1989a,b); *Neotoma micropus* (Garcia and Perez, 1984); *Erinaceus europaeus* (de Witt and Weströmm, 1987); *Herpestes edwardsii* (Tomihara *et al.*, 1987); *Dinodon semicarinatus* (Tomihara *et al.*, 1988); and *Philander opossum* (Domont *et al.*, 1989). The protective antihemorrhagic and antineurotoxic factors have some common characteristics: they are acid proteins with isoelectric points ranging between 4.0 and 5.4; their molecular masses vary from 52 to 90 kDa, with one exception of 780 kDa; none has proteolytic activity; their pH and thermostabilities are high and they seem to be glycoproteins. No precipitation lines are formed between the neutralizing proteins and the venoms upon immunodiffusion, indicating that the serum protective factors are not immunoglobulins. The possible mode of action of the antineurotoxic factor isolated from *Vipera palaestinae* by Ovadia *et al.* (1977) is shortly discussed as well as the possibility that the antihemorrhagic factors may act by a similar mechanism.

## INTRODUCTION

In writing a paper on natural immunity against snake venoms, one is conscious that over 200 years have passed since the pioneering work of Fontana (1781) in which we find his celebrated aphorism: "the venom of the viper is not venomous to its species". Not much has changed in this field until the last 25 years. As a result of this, we will try to review the important steps that took place in the development of main concepts and focus on the purification of protective factors, their properties and mode of action.

---

A difficulty arises from protection studies found in the literature: they report an enormous diversity of animals and venoms. To keep the paper to a reasonable length, we have restricted this field. To many, this can be an unpardonable restriction; to authors, our apologies to those whose contributions we slight.

As a prelude to the rest of the paper, it seems worthwhile to outline the history of natural immunity against snake venoms.

It is certainly true to claim that studies of natural immunity began with FONTANA (1781) who reported the very high resistance of poisonous snakes to their own venom and indeed thought it absolute, that is, independent of the venom dose.

In the 19th century, several concepts were settled down.

1. FONTANA's observations are recognized and natural immunity is postulated not to be absolute (CLAUDE-BERNARD, 1857; DUMÉRIL, 1854; FAYRER, 1874; GUYON, 1861; MITCHEL, 1861; WADDELL, 1889). 2. Inter-specific snakes natural immunity is discovered, probably by GUYON (1861). 3. Natural immunity of other animals, e.g. mongoose (*Herpestes ichneumon*; CALMETTE, 1895) and hedgehog (*Erinaceus europaeus*; PHISALIX and BERTRAND, 1895) is established.

All experiments performed in this period do not inform us about the amount of venom used. Direct bite by a venomous snake was common and inoculated doses were measured as number of drops of a freshly collected venom. It was PHISALIX (1922) who first reported the use "on recent experiments" of weighted amounts of desiccated venoms dissolved in saline.

In the first two decades of the 20th century, natural immunity experiments with other animals than snakes is prevalent. PHISALIX (1922) reports in her classic book "Animaux Venimeux et Venins" tests with invertebrates, fishes, batrachians and mammals.

A huge amount of data is published in the next five decades. Some observations are of a qualitative nature, describing only the resistance of certain animals to snake venoms (e.g. GLOYD, 1933) while others examine the resistance of animals by direct i.m. or i.p. injections of measured quantities of venom calculated per body weight of the tested animals (KEEGAN and ANDREWS, 1942; KELLAWAY, 1937; KUWAJIMA, 1953; PHISALIX, 1922; SWANSON, 1946). Other studies show that some non-venomous snakes also survive after being bitten by viperid or crotalid snakes (ABALOS, 1963; DITMARS, 1966; KLAUBER, 1956).

In the last 25 years increasing attempts have been made to isolate more- or less-crude fractions responsible for the animal resistance. Thus, from the serum of *Lampropeltis getulus floridans*, a preparation that inhibits or activates venom proteases was isolated (PHILPOT and DEUTSCH, 1956); from the serum of *Crotalus adamanteus* a fraction that neutralizes lethal doses of this snake venom in mice was prepared (CLARK and VORIS, 1969); plasmas of *Crotalus atrox* and *Crotalus adamanteus* furnished another isolate that has neutralization capacity against several Crotalid poisons (STRAIGHT *et al.*, 1976); from Crotalidae and Colubridae sera preparations that neutralize crotalid venoms were obtained (PHILPOT JR *et al.*, 1978); from serum of the opossum (*Didelphis marsupialis*) a fraction that protects mice against *Bothrops jararaca* and *Crotalus adamanteus* poison (MOUSSATCHÉ *et al.*, 1978, 1979) and against *B. cotiara, B. alternata, B. jararacussu* and *B. neuwiedi* was isolated (MOUSSATCHÉ and LEONARDI, 1982); and from the serum of *Philander opossum* a preparation protected mice against *B. jararaca* poison (MOUSSATCHÉ and LEONARDI, 1982).

Today, emphasis is centered around the isolation of factors, homogeneous by more stringent criteria, for future biochemical and pharmacological studies. These factors, to be

discussed in detail further, were isolated from the sera of *Trimeresurus flavoviridis* (OMORI-SATOH *et al.*, 1972); *Vipera palaestinae* (OVADIA *et al.*, 1975, 1977; OVADIA, 1978*b*); *Dinodon semicarinatus* (TOMIHARA *et al.*, 1988); *Sigmodon hispidus* (PICHYANGKUL and PEREZ, 1981); *Didelphis virginiana* and *Didelphis marsupialis* (MENCHAGA and PEREZ, 1981; MOUSSATCHÉ *et al.*, 1979, 1980, 1981; PERALES *et al.*, 1986; TARNG *et al.*, 1986); *Neotoma micropus* (GARCIA and PEREZ, 1984); *Erinaceus europaeus* (DE WITT and WESTRÖM, 1987); *Herpestes edwardsii* (TOMIHARA *et al.*, 1987); and *Philander opossum* (DOMONT *et al.*, 1989).

Since our knowledge of proteins has advanced so dramatically and since so many powerful microtechniques have become available, future emphasis will be made on three main interrelated fields: determination of the structures and conformations of proteins that afford protection and to their venom counterparts; postulation of their functions and elucidation of their mode of action.

It is hoped that these developments will have far reaching implications in the treatment of snake bites with the possible goal of applications to humans.

## GENERAL CONCEPTS

A review of the literature on natural immunity shows that comparison of results published by different investigators is not always possible since no standard concepts and assays are used and quantification is not unified. For instance, investigators have used as criteria of resistance the survival of mice after injection of one $LD_{100}$ (MOUSSATCHÉ *et al.*, 1980); two $LD_{50}$ (OVADIA and KOCHVA, 1977); two-and-a-half $LD_{50}$ (PHILPOT and SMITH, 1950); and five $LD_{50}$ (OMORI-SATOH *et al.*, 1972). Inoculation routes have varied also, for example caudal vein (OVADIA and KOCHVA, 1977) or intraperitoneal (PHILPOT and SMITH, 1950).

There are two ways of determining natural immunity: by direct injection of venom into the animal whose resistance is to be assayed or by transference of the protective material from the animal whose resistance is to be tested, be it pure or not, to another animal to be challenged with the venom. The first assay is a measure of direct resistance; the second is a passive resistance or protection test and implies that the resistance of an animal can be transferred to another animal, affording it protection. This is not always the case as shown by experiments with the mongoose (*Herpestes ichneumon*) and *Walterinnesia aegyptia* (OVADIA and KOCHVA, 1977). The first assay, in spite of being direct and more reliable, has two difficulties: the number of specimens needed to complete the assay and the amount of venom to be spent. The second—passive resistance—is routinely performed employing Swiss white mice. Another point to be stressed in the second assay is whether venom and protective material should be inoculated separately or should be injected only after their prior mixture and incubation.

Thus, it seemed very important to the authors to propose a set of definitions that could lead to a standardization of terms, assays and of quantitative data, so that the comparison and evaluation of results obtained by different investigators could be made on a sound basis.

It is fair to point out that the views expressed below are personal and, maybe in their totality, unlikely to be shared by other groups. We are aware of the difficulties they imply.

Direct resistance of an animal is conceptually accepted as the survival of the animal when inoculated intraperitonially with at least four $LD_{50}$ previously determined in Swiss white mice.

Passive resistance of an animal is conceptually accepted as the survival of Swiss white mice when a mixture of the protective material and tw $LD_{50}$, preincubated for 30 min at 37°C, is i.p. inoculated (see direct or passive resistance dose below).

In both cases, recovery from bad health conditions due to envenomation is acceptable and is a sign of resistance.

Neutralization of specific venom properties like hemorrhagic or neurotoxic properties should not be taken as a resistance assay but, rather, as the direct or passive test of the neutralizing power of the material toward a specific venom property.

One direct (DRD) or passive (PRD) resistance dose is defined as the amount of protective material, expressed in micrograms, that neutralizes the lethal action of one $LD_{50}$ when the animal is i.p. inoculated with two $LD_{50}$. In other words, it is the amount of protective material that restores the original venom $LD_{50}$ value in animals inoculated with two $LD_{50}$.

Autologous direct resistance exists when an animal inoculated with its own venom survives or when other specimens of the same species challenged with the species venom survive. Shortly, when there is intra-specific resistance.

Autologous passive resistance exists when Swiss white mice survive the inoculation of a preincubated mixture of the protective material and venom, both collected from the same animal or from other specimens of the same species.

Venom, pooled or not, milked from animals of one species and inoculated in a member of this same species is considered an autologous venom.

Isologous direct resistance exists when an animal of one species survives the inoculation of venom from an animal of another species of the same genus. Shortly, when there is inter-specific resistance in the same genus.

Isologous passive resistance exists when Swiss white mice survive the inoculation of a preincubated mixture of protective material collected from an animal of one species with venom from an animal of another species of the same genus.

Venom milked from animals of one species and inoculated in a member of another species of the same genus is considered an isologous venom.

Homologous direct resistance exists when an animal classified in a genus survives the inoculation of venom obtained from an animal of another genus of the same family. Shortly, when there is intergeneric resistance among specimens of a unique family.

Homologous passive resistance exists when Swiss white mice survive the inoculation of a preincubated mixture of protective material obtained from an animal of a genus with venom from an animal of another genus of the same family.

Venom milked from animals belonging to a genus when inoculated in an animal of another genus is considered a homologous venom.

Heterologous direct resistance exists when an animal classified in a family survives the inoculation of venom obtained from an animal of another family of the same order. Shortly, when there is inter-familial resistance among specimens of the same order.

Heterologous passive resistance exists when Swiss white mice survive the inoculation of a preincubated mixture of protective material obtained from an animal of a family, with venom from an animal of another family of the same order.

Venom milked from animals belonging to a family when inoculated in an animal of another family of the same order is considered a heterologous venom.

Xenologous direct resistance exists when an animal from an order survives the inoculation of venom from an animal of another order of any class. Shortly, when there is inter-orderial resistance among members of any class.

Xen 1 gous passive resistance exists when Swiss white mice survive the inoculation of a preincubated mixture of protective material obtained from an animal of an order with venom from an animal of another order of any class.

Venom milked from animals belonging to an order when inoculated in an animal of another order of any class is considered a xenologous venom.

The same nomenclature used for venoms applies to sera.

Whenever possible the results reported by others were modified to meet our concepts. Due to the lack of data in many papers and to the non-uniformized results found in the literature, our quantitative concept of resistance was not adopted in this work; the original data were used.

## NEUTRALIZING PROTEINS: PURIFICATION AND PROPERTIES

### Antihemorrhagic proteins (AHP)

The first antihemorrhagic proteic factor to be purified and physico-chemically characterized was the one isolated from the serum of a crotalid, *Trimeresurus flavoviridis* (OMORI-SATOH *et al.*, 1972). Its purification needed several steps: ammonium sulfate fractionation (35–60% saturation) followed by cold ethanol fractional precipitation, gel permeation chromatography in Sephadex G-200 and a further ion-exchange DEAE-column step. A final yield of 15% and a purification factor of 21 was obtained.

Its homogeneity was checked by sedimentation velocity, conventional disc electrophoresis, Sephadex G-200, isoelectric focusing and immunoelectrophoresis. All these methods showed the presence of one single band or component. It was characterized as an $\alpha_1$-globulin or an albumin.

The neutralizing protein has a molecular mass of 70 kDa and a partial specific volume of 0.749 cm$^3$/g by sedimentation equilibrium. Isoelectric focusing showed an isoelectric point of 4.0. It is stable between pH 2 and 11 and thermostable, retaining almost all of its antihemorrhagic activity when heated at 56°C for 30 min.

This AHP inhibited, in the depilated back skin of rabbits, the hemorrhagic activities of HR$_1$ and HR$_2$, the two hemorrhagins present in Habu's (*Trimeresurus flavoviridis*) venom.

The inhibition by Habu serum of the hemorrhagic activity of venoms from different species, genera and families of snakes was also assayed. The hemorrhagic activity of autologous and isologous venoms was inhibited more effectively. Homologous venoms were also well inhibited except for the venom of *Bothrops jararaca*. On the other hand, inhibition of the activity of heterologous venoms from Elapidae and Viperidae was fairly poor (OMORI-SATOH, 1972).

An antihemorrhagic protein isolated from the serum of *Vipera palaestinae* (OVADIA, 1978b) neutralized all the hemorrhagic principles previously isolated from the autologous venom by OVADIA (1978a). The purification scheme followed almost identically the one used by OMORI-SATOH *et al.* (1972): precipitation by ammonium sulfate (45–55% saturation) was accompanied by gel permeation (Sephadex G-75), ion-exchange (DEAE-cellulose) and isoelectric focusing (column). The yield was 12% and a purification factor of 5 was achieved. SDS-PAGE, disc electrophoresis and immunoelectrophoresis showed one single band.

Characterization of the antihemorrhagic protein showed the following properties: molecular mass of 80 kDa by SDS-PAGE, isoelectric point of 4.7 by IEF, glycoprotein nature by Schiff's staining, stability between pH 4 and 9.5 and thermostability at 85°C for 10 min. It had n caseinase or gelatinase activities.

This factor was able to neutralize the three hemorrhagic fractions of *Vipera palaestinae* venom (OVADIA, 1978*a,b*) and five micrograms of the AHP neutralized one minimum hemorrhagic dose (MHD) of the viperids *Echis colorata* and *Cerastes cerastes* homologous venoms.

A factor with xenologous resistance is present in the sera of the opossum species *Didelphis marsupialis* and *Didelphis virginiana*. Two groups using different strategies isolated this protein.

The preparation obtained by MOUSSATCHÉ *et al.* (1978, 1979, 1980, 1981, 1982) and YATES *et al.* (1979) were substituted by a purer fraction (Perales *et al.*, 1986, 1989*a*) prepared using a strategy simpler than its predecessors. It employs an adaptation of Shibata's technique (1977) for the isolation of acid-$\alpha_1$-glycoproteins from sera. Using only DEAE-Sephacel chromatography they were able to isolate from *Didelphis marsupialis* serum a homogeneous fraction with anti-bothropic activity. SDS-PAGE—Coomassie brilliant blue and Schiff's stainings—revealed the presence of two major glycoproteins of molecular masses of 48.5 and 42.6 kDa and a minor contaminant of 55.3 kDa. HPLC-gel permeation chromatography (TSK-G 3000, cut-off 300 kDa) had a profile of a single major peak (83.7 kDa) and two very minor contaminants. These data suggest that the anti-venom fraction is a complex composed of two different subunits, gp 48.5 and gp 42.6 (PERALES *et al.*, 1989*b*). As a first approach for the isolation of the AHP from the serum of *Didelphis virginiana*, MENCHAGA and PEREZ (1981) tried a strategy similar to that used by OMORI-SATOH *et al.* (1972) for the purification of the AHP from the serum of *Trimeresurus flavoviridis*, that is: Sephadex G-200, DEAE-chromatography and permeation in Sephadex G-100. A purification factor of 6.4 was reached. Conventional disc electrophoresis and isoelectric focusing yielded a single band. AHP has a molecular mass by gel permetation of 68 kDa, an isoelectric point of 4.1, no gelatinase activity, pH stability between 2 and 10 and is thermostable between 0 and 37°C. It neutralized all five hemorrhagic toxins present in *Crotalus atrox* venom.

A second approach was to use this AHP to produce a specific monoclonal antibody. Immunoaffinity chromatography showed that the AHP could be isolated using its antibody (TARNG *et al.*, 1986). Although conventional slab gel electrophoresis revealed one protein band, two bands—heavy and faint—were revealed in SDS-PAGE and isoelectric focusing, with molecular masses of 65 and 57 kDa and isoelectric points of 4.8 and 4.1, respectively. The authors suggest that some molecules could be reduced by mercaptoethanol present in the sample buffer and migrate to a different position in the SDS-PAGE gel.

Serum of *Neotoma micropus* was the source of another xenologous AHP. Employing the same rationale used to *Didelphis virginiana* serum, GARCIA and PEREZ (1984) purified a factor with antihemorrhagic specific activity against *C. atrox* venom 8.9 times that of the original woodrat crude serum that showed one single band by conventional disc-PAGE in the globulin region. It has an isoelectric point of 4.1 by column isoelectric focusing and a molecular mass of 54 kDa by gel permeation column chromatography. Activity was retained between 0 and 56°C and in 3–10 pH range. No proteolytic activity was found when gelatin and hide powder were tested as substrates. No precipitate was formed between AHP and crude venom, that is, there occurred no binding in a polyvalent manner. Taken together with its physical characteristics, it is suggested that AHP is n t a γ-globulin.

A xenologous AHP with activity against *Vipera berus* venom and with chemical properties different from those of other known factors was isolated from the plasma f the

hedgehog, *Erinaceus europaeus* (DE WITT and WESTRÖM, 1987a,b). Using a method 1 gy developed for the isolation of macroglobulins—Sephacryl S-200 and Cibacron Blue Sepharose chromatographies—these investigators were able to purify a protein fraction that showed a major band in the $\alpha_2$-region and two minor bands in the $\alpha_2$-$\beta$ and $\beta$-regions by agarose gel electrophoresis and one major (780 kDa) and three weak bands (670, 550 and 539 kDa) by gradient (2–16%) polyacrylamide gel electrophoresis. This combined fraction of hedgehog macroglobulin ($\alpha_2$-, $\alpha_2$-$\beta$ and $\beta$-macroglobulins) was shown to totally neutralize the hemorrhagic activity of *Vipera berus* venom. All three agarose bands had inhibiting activity against trypsin, chymotrypsin, elastase, collagenase, papain and plasmin whereas preliminary results showed that the major PAGE band and at least one of the bands in the 540–550 kDa range inhibit trypsin.

Immunoelectrophoretically, the $\alpha_2$-protein cross-reacts with anti-serum to human $\alpha_2$-macroglobulin, swine $\alpha_2$-macroglobulin (fast) and rat $\alpha_2$-acute phase globulin, while the $\alpha_2$-$\beta$- and $\beta$-proteins cross-react with swine $\alpha_2$-macroglobulin (slow).

Another xenologous factor was isolated from the serum of the hispid cotton rat, *Sigmodon hispidus*, using a linear salt gradient to elute a DEAE-Sephadex column followed by flat-bed electrofocusing conducted in Sephadex G-75 (PICHYANGKUL and PEREZ, 1981). Final yield was 14% with a purification factor of 20. Conventional disc electrophoresis of the purified factor gave one single band and sucrose gradient centrifugation revealed a symmetrical single peak. This AHP has a molecular mass of 90 kDa by gel filtration in Sephadex G-200 and of 56 kDa calculated from a sedimentation coefficient of 4.2. These data suggest that the molecule is elongated. An isoelectric point of 5.4 was calculated from the flat bed experiment. No collagenase or gelatinase activities were detected; pH and thermostabilities ranged between 3–10 and 0–55°C, respectively. This AHP neutralizes the five reported hemorrhagic activities present in the venom of *Crotalus atrox* venom.

Recently (DOMONT *et al.*, 1989) a protein fraction present in the serum of *Philander opossum* and responsible for its resistance to *Bothrops jararaca* venom was isolated using the same methodology applied to *Didelphis marsupialis* serum (PERALES *et al.*, 1989a,b). Its purity checked by HPLC-gel permeation (TSK-G 3000) showed the presence of two main peaks of 82.5 and 66.2 kDa and by SDS-PAGE, again, two main fractions were stained by Coomassie brilliant blue and Schiff's reagent with molecular masses of 48.5 and 42.6 kDa. In both cases, several minor contaminants were detected. Like the active proteic complex isolated from *Didelphis marsupialis* serum, the *Philander opossum* DEAE-fraction was shown to be composed of two subunits. Their relative mobilities were identical to those of the subunits isolated from *D. marsupialis* serum.

Three xenologous AHP were also isolated from the serum of a mongoose (*Herpestes edwardsii*) by TOMIHARA *et al.* (1987). After Sephadex G-200 chromatography, concentration by ultrafiltration and dialysis, the active fractions were submitted to four successive HPLC-chromatographies employing a preparative TSK-DEAE-5PW column and varying gradient curves and/or buffer. AHP-1, AHP-2 and AHP-3 were purified to 66.1–69.4 and 74.7-fold in yields of 10.5%, 8.4% and 7.4%, respectively. All three AHP were homogeneous by conventional polyacrylamide gel electrophoresis: AHP-1 was the most acidic and AHP-2 and AHP-3 showed the same relative mobility. These proteins had the same molecular mass of 65 kDa by gel permeation chromatography whereas by reductive SDS-PAGE a single 69 kDa band was found.

These proteins were able to equally neutralize the action of $HR_1$ and $HR_2$ the two hemorrhagins present in *T. flavoviridis* venom. They were stable between pH 2 and 11,

from 0 to 60°C at pH 7.0 for 15 min and formed no precipitin lines against $HR_1$, $HR_2$  r whole venom. The three antihemorrhagic factors of *H. edwardsii* may be the first evidence for the presence of different AHP against a snake venom in an animal serum.

These investigators (TOMIHARA *et al.*, 1988) now using only five successive HPLC-chromatographies in the same TSK-DEAE-5PW preparative column and again employing different gradient curves and/or buffer in each column cycle, purified another AHP from the serum of a non-venomous snake (*Dinodon semicarinatus*). This time a purification factor of 30.3 and a yield of 2.8% were obtained. Only one band was stained after conventional PAGE and a single 52 kDa protein was shown to be present after SDS-PAGE. As was shown with the three AHP from *H. edwardsii*, this antihemorrhagic factor neutralized $HR_1$ and $HR_2$ from *T. flavoviridis*, was stable between pH 2 and 11 and from 0 to 60°C at pH 7.0 for 15 minutes and did not form precipitin lines with the same antigens.

### Antineurotoxic proteins (ANP)

Purification of an antineurotoxic protein from *Vipera palaestinae* serum was accomplished by the classical scheme of OMORI-SATOH *et al.* (1972): ammonium sulfate fractionation (40–55% saturation), gel permeation in Sephadex G-75 and DEAE-cellulose chromatography (OVADIA *et al.*, 1977).

Homogeneity of this ANP by IEF showed a single component with pI 4.0 and its molecular mass determined by SDS-PAGE gave a value of 56 kDa. This factor is thermostable at 70°C for 10 min, but its neutralizing effect is totally abolished by heating for 10 min at 90°C.

ANP forms an inactive complex with *V. palaestinae*'s neurotoxin. When this neutralized complex, now made using whole serum, was acidified to pH 2.5 and back titrated to a neutral pH, neurotoxic activity was regained. This seems to indicate that the neutralizing protein is acid sensitive and that the neurotoxin is not irreversibly inactivated, destroyed or degraded by the serum protein.

### COMPARISON OF PROPERTIES OF THE ACTIVE PROTEINS

In Table 1 we list the properties of the hemorrhagic and neurotoxic proteins. From the text and Table 1 several common features are evident: neutralizing proteins are acidic and have reported isoelectric points between 4.0 and 5.4; the molecular masses of the AHP vary between 52 and 90 kDa with one exception at 780 kDa, whereas that of the ANP is 56 kDa; none has proteolytic activity; in one case AHP has proteolytic inhibitory activities; active complexes made of two different subunits are present in *D. marsupialis* and *P. opossum*; their pH and thermostability are high, reported extremes ranging from 2 to 11 and 0 to 80°C, respectively; they proved to induce, in the recipient animal, autologous resistance, as is the case for AHP from *Trimeresurus flavoviridis* and *Vipera palaestinae*; heterologous resistance as is the case for *Dinodon semicarinatus* as well as xenologous resistance as shown by *Didelphis marsupialis* and *virginiana*, *Sigmodon hispidus*, *Philander opossum*, *Neotoma micropus*, *Erinaceus europaeus* and *Herpestes edwardsii*; they als  seem to be glycopr teins; n  precipitate is formed between the neutralizing proteins and the venom upon immunodiffusion; and as proteins, they were classified as albumin, albumin-like, macroglobulin, α-globulin,  r more specifically as $α_1$-glycoprotein.

TABLE 1. PROPERTIES OF ANTIHEMORRHAGIC AND ANTINEUROTOXIC PROTEINS

| Properties | Species | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | T.f. | V.p. | S.h. | D.m. | D.v. | N.m. | E.e. | H.e. | D.s. | P.o. | V.p.* |
| Molecular mass (kDa) | 70 | 80 | 56/90 | 83.7 | 68 | 54 | 780 | 65–69 | 52 | 82.5/66.2 | 56 |
| Subunits: | | | | | | | | | | | |
| number | — | — | — | 2 | — | — | — | — | — | 2 | — |
| molecular mass (kDa) | — | — | — | 48.5 42.7 | — | — | — | — | — | 48.5 42.6 | — |
| Isoelectric point | 4.0 | 4.7 | 5.4 | 4.0 | 4.1 | 4.1 | — | — | — | — | 4.0 |
| Thermostability (°C) | 0–56 | 0–85 | 0–55 | — | 0–37 | 0–56 | — | 0–60 | 2–11 | — | 0–70 |
| pH stability | 2–11 | 4–9.5 | 3–10 | — | 3–10 | 3–10 | — | 2–11 | 0–60 | — | † |
| Sedimentation coefficient | 4.5 | — | 4.2 | — | — | — | — | — | — | — | — |
| Proteolytic activity | — | no | no | no | no | no | — | — | — | — | — |
| Glycoprotein | — | yes | — | yes | — | — | — | — | — | yes | no |
| Ring precipitin test | no ppt | no ppt | no ppt | no ppt | no ppt | no ppt | — | no ppt | no ppt | — | no ppt |
| Protease inhibitor | — | — | — | — | — | — | yes | — | — | — | — |
| Electrophoretic mobility | α₁/ab | α₁ | α₁. | α₁ | albumin | α₁ | α₁,α-β,β | — | — | α₁ | α₁ |

T.f., *Trimeresurus flavoviridis* (OMORI-SATOH *et al.*, 1972); V.p., *Vipera palaestinae* (OVADIA, 1978b); S.h., *Sigmodon hispidus* (PICHYANGKUL and PEREZ, 1981); D.m., *Didelphis marsupialis* (PERALES *et al.*, 1989a); D.v., *Didelphis virginiana* (MENCHAGA and PEREZ, 1981); N.m., *Neotoma micropus* (GARCIA and PEREZ, 1984); E.e., *Erinaceus europaeus* (DE WITT and WESTRÖM, 1987); H.e., *Herpestes edwardsii* (TOMIHARA *et al.*, 1987); D.s., *Dinodon semicarinatus* (TOMIHARA *et al.*, 1988); P.o., *Philander opossum* (DOMONT *et al.*, 1989); V.p.*, *Vipera palaestinae* (OVADIA *et al.*, 1977), antineurotoxic; † unstable at pH 2.5; —, not determined.

## MODE OF ACTION

Historically, researches were directed to find out whether resistance of an animal is a result of tolerance of its tissues (KELLAWAY, 1937); to the presence of inherited antibodies or to antibodies acquired by immunization in the field (BOQUET, 1945; MIRANDA, 1982; PHISALIX, 1922) or to presence of inherited humoral antitoxins found in their blood.

That tolerance of tissues is not a probable mechanism of protection was shown  y KUWAJIMA (1953). ROSENFELD and GLASS (1940) suggest that the neutralizing phenomenon is not that of a specific antibody reaction, but rather, the result of a non-specific neutralization of venom hemorrhagins. This is also suggested by the non-formation of a precipitation line upon immunodiffusion against snake venoms and by the chemical and physicochemical properties of the isolated neutralization proteins as shown in Table 1. VELLARD (1945, 1949) raised the hypothesis of inherited factors and PERALES (1986) showed that newborn *Didelphis marsupialis* reared up in the laboratory and separated from the mother before maternal feeding had natural immunity against *B. jararaca* venom.

The mode of action of the neutralizing proteins has been studied only superficially. The existence of a common humoral mechanism of inhibition of the hemorrhagic activity of snake venoms (OMORI-SATOH *et al.*, 1972) and its possible relationship of the mode of neutralization of the neurotoxic activity of *Vipera palaestinae* venom has been suggested (OVADIA, 1987*b*). However, it is not even clear from the literature whether there is really a common humoral mode of action of the antihemorrhagic proteins as a group or when compared to that of the antineurotoxic activity of *Vipera palaestinae*.

Nevertheless, the studies of OVADIA *et al.* (1977) shed some light on the humoral mechanism of neutralization of *V. palaestinae* neurotoxin by the protein isolated from autologous serum. An *in vivo* time-dependent experiment showed that the ANP does not block the neurotoxin target sites permanently. Rather, it forms a thermostable complex with the neurotoxin as evidenced by Sephadex G-75 chromatography using ANP and neurotoxin labelled with $^{131}$I.

The neurotoxic fraction of *V. palaestinae* can be separated into four components by column isoelectric focusing. None killed mice. However, two components of isoelectric points of 9.5 and 4.0 had synergistic activity with the characteristics lethal effects of the whole neurotoxic fraction. Neurotoxic component of pI 9.0 seems to bind non-specifically to ANP whereas ANP interaction with the pI 4.0 component is suggested as a specific reaction. Since the experiments were not performed on a molar ratio basis and considering that ANP also has a pI of 4.0 the nature of the interaction between the proteins that form the complex remains unclear.

Acccording to the investigators, their "findings point to a neutralization mechanism which is different from the well known immune reaction but which behaves in a similar fashion to detoxify and remove toxic materials made by the host's own tissues. Unlike the immune response, the mechanism involves an acidic and comparatively low molecular weight protein that form a stable complex".


## PERSPECTIVES

Before we can use  ur knowledge of natural immunity processes to try to achieve the ultimate goal  f applicati ns to humans, if it proves feasible, much remains to be searched f r and n t much will be kn wn in the near future. Isolati n  f proteins with neutralizing

activities and f their ven m counterparts is important and necessary but not sufficient, *per se*, t allow the postulation f a neutralizati n mechanism. This shall begin to be elucidated at the molecular level only after we determine: 1. the chemical structures of the important proteins; 2. the conformation of each protein and of its complex; 3. the physicochemical parameters of each protein and of their interactions and; 4. the biological action of pure venom proteins, of whole venoms and the biological role of neutralizing proteins.

Only then we shall attempt to understand the semantics of what we denote as natural immunity.

## REFERENCES

ABALOS, J. W. (1963) The ophiofagus habits of *Pseudoboa cloelia*. *Toxicon* **1**, 90–91.

BOQUET, P. (1945) Sur les propriétés antivenimeuses du serum de *Vipera aspis*. *Ann. Inst. Pasteur* **71**, 340–343.

CALMETTE, A. (1895) Experiences sur l'immunité de la mangouste. *Ann. Inst. Pasteur*, Avril; apud Phisalix, M. (1922).

CLARK, C. W. and VORIS, K. H. (1969) Venom neutralization by rattlesnake serum albumin. *Science* **164**, 1402–1404.

CLAUDE-BERNARD (1857) Leçons sur l'effet des substances toxiques; apud Kellaway, C. H. (1937).

DE WITT, C. A. and WESTRÖM, B. R. (1987a) Venom resistance in the hedgehog (*Erinaceus europaeus*) purification and identification of macroglobulin inhibitors as plasma antihemorrhagic factors. *Toxicon* **25**, 315–323.

DE WITT, C. A. and WESTRÖM, B. R. (1987b) Purifications and characterization of $\alpha_1$-, $\alpha_2$-$\beta$ and $\beta$-macroglobulin inhibitors in the hedgehog, *Erinaceus europaeus*: -macroglobulin identified as the plasma antihemorrhagic factor. *Toxicon* **25**, 1209–1219.

DITMARS, R. L. (1966) *Reptiles of the World*, pp. 182–183. New York: Macmillan.

DOMONT, G. B., PERALES, J. and MOUSSATCHÉ, H. (1989) Partial purification and physico-chemical characterization of a proteic complex from *Philander opossum* with protective action against *Bothrops jararaca* venom. Brazilian–Sino Symposium on Chemistry and Pharmacology of Natural Products, 10–14 December, Rio de Janeiro, Brazil.

DUMÉRIL, A. (1854) Notice historique sur la ménagerie des reptiles du Museum d'Hist. Nat. *Arch. du Mus.* **VII**, 273; apud Phisalix, M. (1922).

FAYRER, J. (1874) *The Thanatophidia of India*, 2nd Edn; apud Kellaway, C. H. (1937).

FONTANA, F. (1781) *Traité sur le venin de la Vipère*. Florence, p. 23; apud Phisalix, M. (1922).

GARCIA, V. E. and PEREZ, J. G. (1984) The purification and characterization of an antihemorrhagic factor in woodrat (*Neotoma micropus*) serum. *Toxicon* **22**, 129–138.

GLOYD, H. K. (1933) On the effects of mocassin venom upon a rattlesnake. *Science* **78**, 13–14.

GUYON, J. (1861) Le venin des Serpents exerce-t-il sur eux-mêmes l'action qu'il exerce sur d'autres animaux? C. R. Ac. des Sc. LIII, 12; apud Phisalix, M. (1922).

KEEGAN, H. L. and ANDREWS, T. F. (1942) Effects of crotalid venom on North American snakes. *Copeia* **4**, 251–254.

KELLAWAY, C. H. (1937) Snake venoms. III. Immunity. *Bull. Johns Hopkins Hosp.* **60**, 159–177.

KLAUBER, L. M. (1956) *Rattlesnakes*, pp. 853–858. Berkeley, San Diego: University of California Press.

KUWAJIMA, Y. (1953) Immunological researches on the main Formosan poisonous snakes, especially on the venoms. *Jpn. J. Exp. Med.* **23**, 465–471.

MENCHACA, J. M. and PEREZ, J. C. (1981) The purification and characterization of an antihemorrhagic factor in opossum (*Didelphis virginiana*) serum. *Toxicon* **19**, 623–632.

MIRANDA, M. E., COUTURIER, G. A., CANZIANI, A., SEKI, C. and VIDAL, J. C. (1982) Resistencia de *Crotalus durissus terrificus*, *Bothrops neuwiedii* a la neurotoxicidad de cantidades masivas de veneno crotálico. *Acta Physiol. latinoamericana* **32**, 103–115.

MITCHELL, W. (1861) Physiology and toxicology of the venom of the rattlesnake. Smithsonian Contributions to Knowledge; apud Kellaway, C. H. (1937).

MOUSSATCHÉ, H. and LEONARDI, F. (1982) Estudios de protección con suero de mamiferos y reptiles a los venenos de serpientes Crotalidae. *Acta cient. venezolana* **33** (sup. 1), 151.

MOUSSATCHÉ, H., LEONARDI, F. and MANDELBAUM, F. (1981) Inhibición por una proteina aislada del suero de *D. marsupialis* a la acción hemorrágica por una fracción del veneno de *Bothrops jararaca*. *Acta cient. venezolana* **32** (sup. 1), 173.

MOUSSATCHÉ, H., YATES, A., LEONARDI, F., YATES, A. and BORCHE, L. (1978) Experimentos sobre la resistencia del Didelphis venezolano (rabi-pelado) a los venenos de serpientes. Acta cient. venezolana 29 (supl. 1), 55.

MOUSSATCHÉ, H., YATES, A., LEONARDI, F. and BORCHE, L. (1979) Mechanisms of resistance of the opposum to some snake venoms. Toxicon 17, 130.

MOUSSATCHÉ, H., YATES, A., LEONARDI, F. and BORCHE, L. (1980) Obtención de una fracción del suero de Didelphis activa contra la acción tóxica del veneno de B. jararaca. Acta cient. venezolana 31 (suppl. 1), 104.

OMORI-SATOH, T., SADAHIRO, S., OHSAKA, A. and MURATA, R. (1972) Purification and characterization of an antihemorrhagic factor in the serum of Trimeresurus flavoviridis, a crotalid. Biochim. biophys. Acta 285, 414-426.

OVADIA, M. (1978a) Isolation and characterization of three hemorrhagic factors from the venom of Vipera palaestinae. Toxicon 16, 479-487.

OVADIA, M. (1978b) Purification and characterization of an antihemorrhagic factor from the serum of the snake Vipera palaestinae. Toxicon 16, 661-672.

OVADIA, M. and KOCHVA, E. (1977) Neutralization of Viperidae and Elapidae snake venoms by sera of different animals. Toxicon 15, 541-547.

OVADIA, M., KOCHVA, E. and MOAV, B. (1977) The neutralization mechanism of Vipera palaestinae neurotoxin by a purified factor from homologous serum. Biochim. biophys. Acta 491, 370-386.

OVADIA, M., MOAV, B. and KOCHVA, E. (1975) Factors in the blood serum of Vipera palaestinae neutralizing fractions of its own venom. Toxicon 13, 113.

PERALES, J., MUÑOZ, R. and MOUSSATCHÉ, H. (1986) Isolation and partial characterization of a protein fraction from the opossum (Didelphis marsupialis) serum, with protecting property against the Bothrops jararaca snake venom. An. Acad. brasil. Ciênc. 58, 155-162.

PERALES, J., MUÑOZ, R., GRATEROL, A., OVIEDO, O. and MOUSSATCHÉ, H. (1989a) New findings on the purification and characterization of an anti-bothropic factor from Didelphis marsupialis (opossum) serum. Brazilian J. Med. Biol. Res. 22, 25-28.

PERALES, J., DOMONT, G. B. and MOUSSATCHÉ, H. (1989b) Physicochemical characterization of the antibothropic fraction from Didelphis marsupialis serum. Brazilian-Sino Symposium on Chemistry and Pharmacology of Natural Products, 10-14 December, Rio de Janeiro, Brazil.

PHILPOT, V. B. and SMITH, R. G. (1950) Neutralization of pit viper venom by king snake serum. Proc. Soc. exp. Bio. Med. 75, 521-523.

PHILPOT, JR, V. B. and DEUTSCH, H. F. (1956) Inhibition and activation of venom proteases. Biochim. biophys. Acta 21, 524-530.

PHILPOT, JR, V. B., EZEKIEL, E., LASETER, Y., YAEGER, R. G. and STJIRNHOLM, R. L. (1978) Neutralization of crotalid venoms by fractions from Snake Sera. Toxicon 16, 603-609.

PHISALIX, C. and BERTRAND, G. (1895) Sur l'immunité du Hérisson contre le venin de Vipère (Rep. à Lewin). C. R. Soc. Biol. XLIII, p. 639. Apud Phisalix, M. (1922).

PHISALIX, M. (1922) Animaux Venimeux et Venins, pp. 744-759. Paris: Mason & Cie. Editeurs.

PICHYANGKUL, S. and PEPUZ, J. G. (1981) Purification and characterization of a naturally occurring antihemorrhagic factor in the serum of the hispid cotton rat (Sigmodon hispidus). Toxicon 19, 205-215.

ROSENFELD, S. and GLASS, S. (1940) The inhibiting effect of snake blood upon the hemorrhagic action of viper venoms on mice. Am. J. Med. Sci. 199, 482-486.

SHIBATA, K., OKUBO, H., ISHAIBASHI, H. and TSUDA, K. (1977) Rat α-acid glycoprotein purification and immunologocal estimation of its serum concentration. Biochim. biophys. Acta 495, 37-45.

STRAIGHT, R., GLENN, G. L. and SNYDER, C. C. (1976) Antivenom activity of rattlesnake blood plasma. Nature 261, 259-260.

SWANSON, P. L. (1946) Effects of snake venoms on snakes. Copeia 4, 242-249.

TARNG, S. F., HUANG, S. Y. and PEREZ, J. C. (1986) Isolation of antihemorrhagic factors in opossum (Didelphis virginiana) serum using a monoclonal antibody immunoadsorbent. Toxicon 24, 567-573.

TOMIHARA, Y., YONAHA, K., NOZAKI, M., YAMAKAWA, M., KAMURA, T. and TOYAMA, S. (1987) Purification of three antihemorrhagic factors from the serum of a mongoose (Herpestes edwardsii). Toxicon 25, 685-689.

TOMIHARA, Y., YONAHA, K., NOZAKI, M. YAMAKAWA, M., KAWAMURA, Y., KAMURA, T. and TOYAMA, S. (1988) Purification of an antihemorrhagic factor from the serum of the non-venomous snake (Dinodon semicarinatus). Toxicon 26, 420-423.

VELLARD, J. (1945) Resistencia de los "Didelphis" (Zarigueya) a los venenos ofidicos (Nota previa). Rev. Brasil. Biol. 5, 463-467.

VELLARD, J. (1949) Investigaciones sobre inmunidad natural contra los venenos de serpientes. I. Publnes. Mus. Hist. nat., Lima Ser. A., Zool. 1, 1-61.

WADDELL, L. A. (1889) Are venomous snakes autotoxic? An enquiry into the effects of serpent venoms upon the serpents themselves. Scientific Memoirs by Medical Officers of the Army of India, part IV, 47; apud Kellaway, C. H. (1937).

YATES, A., MOUSSATCHÉ, H., LEONARDI, F. and BORCHE, L. (1979) Aislamento de la fracción del suero de Didelphis (rabi-pelado) protectora contra la toxicidad del veneno de B. jararaca. Acta cient. venezolana 30 (supl. 1), 5.